Comparison of different anion-exchange chromatography resins for the purification of cyanobacterial microcystins

Theerasak Somdee and Anchana Somdee

ABSTRACT

For the first time, different types of diethylaminoethyl (DEAE) anion-exchange resins, widely used in previous studies, were investigated to determine the most effective resin for the purification of microcystins (MCs). MCs were extracted from freeze-dried Microcystis aeruginosa cells that had been harvested from the Bueng Nong Khot reservoir, Khon Kaen, Thailand. The toxins were precipitated with ammonium sulfate and then fractionated using five different anion-exchange chromatography resins, followed by chromatography with a C18 cartridge. The toxins were further identified via liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) analysis, and the yields and purity were determined by high-performance liquid chromatography (HPLC) with ultraviolet detection. DEAE Sephadex A-25 exhibited the best overall performance for MC purification regarding both yield and purity, followed by DEAE cellulose, DEAE Sephacel, DEAE Sepharose Fast Flow and Toyopearl DEAE. Four MC variants, MC-RR, MC-FR, [Dha7]MC-LR and MC-WR, were obtained, and [Dha7]MC-LR was the major variant, with a total yield of 53.08 mg and a purity of 95% using the Sephadex resin. This study indicates that protein precipitation and single-column chromatography using DEAE Sephadex A-25 constitute an effective method for the purification of a wide range of MC variants.

Key words | anion-exchange chromatography, DEAE Sephadex A-25 resin, microcystins

INTRODUCTION

Blooms of toxic cyanobacteria are frequently observed in eutrophic water bodies throughout the world (Oudra & Andaloussi 2009). Many bloom-forming species are able to produce secondary metabolites that are toxic to humans and animals. Several genera, such as Microcystis, Anabaena, Planktothrix, Nostoc, and Anabaenopsis, are known to produce cyclic peptide toxins, namely microcystins (MCs) (van Apeldoorn et al. 2007). These toxins primarily affect the liver, causing minor to widespread damage depending on the amount of toxin absorbed (Azevedo et al. 2002). The toxicity of MCs is due to the inhibition of the serine/threonine protein phosphatase enzymes 1 and 2A, resulting in the disruption of many cellular control mechanisms (van Apeldoorn et al. 2007). Due to the biochemical mode of action of MCs, these toxins are thought to be contributing factors in primary liver cancer and subsequently have been demonstrated to exhibit tumor-promoting activity (Butler et al. 2009).

MCs possess the general structure cyclo-(d-Ala1-X2-d-MeAsp3-Z4-Adda5-D-Glu6-Mdha7), where X and Z are variable L-amino acids; d-MeAsp is d-erythro-β-methylaspartic acid, Adda is (2S,5S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid, and Mdha is N-methyl-dehydroalanine (Gehringer et al. 2005). More than 100 MC structural variants have been identified to date (Vesterkvist et al. 2012). These structural variants differ primarily in the two variable L-amino acids (denoted X and Z) and in the methylation or demethylation of MeAsp and Mdha (Fastner et al. 2002). The toxins are named accordingly; for example, in the common MC, MC-LR, L refers to leucine in position X, and R refers to arginine in position Z (Gehringer et al. 2005).
The increasing interest in MCs, for use as analytical standards and as research materials for investigating their toxicity and biodegradation, has greatly increased the demand for high-purity MCs (Ramanan et al. 2000; Phelan & Downing 2007). However, at present there is no consensus on the most efficient method for purifying MCs (Lawton & Edwards 2001). A wide range of chromatographic approaches, such as size exclusion, ion exchange and thin-layer chromatography, have been investigated for MC purification. Because the net charge of an MC is negative at most pH values, a positively charged anion resin (diethylaminoethyl, DEAE) has been commonly used with different ion-exchange resins (Saito et al. 2002; Lee 2009). The first method that utilized anion-exchange (DEAE Sephadex A-25) column chromatography was documented by Murthy & Capindale (1970) and by Elleman et al. (1978). In another study, ion-exchange DEAE-cellulose was used to purify MC variants (Botes et al. 1982) and Gregson & Lohr (1983) successfully purified a number of MCs from *M. aeruginosa* with DEAE-Sepharose and C18 high-performance liquid chromatography (HPLC). Finally, employing DEAE Toyopearl chromatography and solid-phase extraction cartridges, MC-LR, MC-LY, MC-LF, and MC-LW have been obtained (Saito et al. 2002). In the present study, different types of DEAE anion resins were assessed to determine the best ion exchange resin for purifying adequate quantities of MCs from natural blooms and/or cultured cells.

**MATERIALS AND METHODS**

**Cyanobacterial cells**

A large mass of cyanobacterial (*Microcystis aeruginosa*) cell material was harvested from the littoral zone of the Bueng Nong Khot reservoir, Khon Kaen, Thailand with a 1,000-μm plankton net between February and July 2010. The material was lyophilized and stored at −20 °C until extraction.

**Extraction of the lyophilized material**

The cyanobacterial material was extracted and purified using a modified version of the method used by Saito et al. (2002) (Figure 1). Briefly, for each extraction, 50 g of the freeze-dried material was mixed with 1 L of a 75% (v/v) methanol (MeOH)-0.1% trifluoroacetic acid (TFA) solution and sonicated for 30 minutes. The extract was centrifuged, and the pellets were further extracted twice. The supernatants were pooled, filtered through a GF/C glass filter (Whatman, Maidstone, Kent, UK) and then evaporated to dryness. The residue was re-suspended in 500 mL of distilled water and precipitated by adding ammonium sulfate (at 55% of the saturation concentration). The suspension was stirred at 4 °C overnight, centrifuged, and decanted. The pellet was suspended in 100 mL of MeOH and filtered through a GF/C filter. The filtrate was evaporated to dryness, and the residue was dissolved in 0.05 M MES (2-morpholinoethanesulfonic acid)-KOH (pH 5.5)-20% (v/v) ethanol (EtOH) (solution A). Then, the residue was adjusted to a concentration of 500 mg/mL using solution A.

**Anion-exchange chromatography**

The anion exchangers used in this study were DEAE Cellulose (Whatman, UK), DEAE Sephacel (GE Healthcare, Sweden), DEAE Sephadex A-25 (GE Healthcare, Sweden), DEAE Sepharose Fast Flow (GE Healthcare, Sweden) and Toyopearl DEAE-650 M (Tosoh Bioscience, Japan). Table 1 provides relevant properties of the anion-exchange resins that were evaluated. An equal volume (10 mL) of the extract was loaded onto each of the columns (20 × 4.0 cm i.d.) packed with the resins described above. The toxins were separated with a gradient from solution A [0.05 M MES-KOH (pH 5.5)-20% (v/v) EtOH] to solution B [0.05 M MES-KOH (pH 5.5)-20% (v/v) EtOH-1 M NaCl] at a flow rate of 1 mL/min. Fractions (10 mL each) were collected, and the absorbance was measured at 238 nm.

**Solid-phase extraction (SPE)**

Desalting of the anion-exchange chromatography fractions using SPE cartridges was employed to eliminate salts introduced by the mobile phase. The pooled fractions obtained from the anion-exchange chromatography were passed through Sep-Pak Vac 35 cc (10 g) C18 cartridges (Waters, Ireland) that had been conditioned with 100% MeOH and deionized water. The impurities were removed by successively washing with deionized water and 20% (v/v) MeOH.

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**Table 1:** Properties of the anion-exchange resins evaluated.

<table>
<thead>
<tr>
<th>Resin Type</th>
<th>Core Material</th>
<th>Column Size</th>
<th>Porosity</th>
<th>Cross-Linkage</th>
<th>pH Range</th>
<th>Buffer</th>
<th>Linear Range (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE Cellulose</td>
<td>DEAE</td>
<td>20 × 4.0 cm</td>
<td>50%</td>
<td>5%</td>
<td>5.5-7.0</td>
<td>MES-KOH</td>
<td>0.05-5.0</td>
</tr>
<tr>
<td>DEAE Sephacel</td>
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</tr>
<tr>
<td>DEAE Sepharose Fast Flow</td>
<td>DEAE</td>
<td>20 × 4.0 cm</td>
<td>50%</td>
<td>5%</td>
<td>5.5-7.0</td>
<td>MES-KOH</td>
<td>0.05-5.0</td>
</tr>
<tr>
<td>Toyopearl DEAE-650 M</td>
<td>DEAE</td>
<td>20 × 4.0 cm</td>
<td>50%</td>
<td>5%</td>
<td>5.5-7.0</td>
<td>MES-KOH</td>
<td>0.05-5.0</td>
</tr>
</tbody>
</table>
MeOH, and the toxins were eluted with 70% (v/v) MeOH at a flow rate of 1 mL/min with the compression module.

**Identification of MCs via liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) analysis**

The toxins eluted from the SPE cartridges were identified and characterized via LC-ESI-MS analysis. The LC-ESI-MS/MS system consisted of a Waters Alliance 2,695 liquid chromatography–diode array detector (LC-DAD) (Waters, USA) and a Q-TOF 2 (quadrupole mass filter–time-of-flight) mass spectrometer with a Z-spray ES source (Micromass, UK). All of the fractions were subjected to separation using a 5 μm, 3.9 × 150 mm Symmetry C18 column (Waters, USA) with a particle size of 3.5 μm. The mobile phase consisted of acetonitrile (solvent A) and 0.1% (v/v) formic acid in water (solvent B). The gradient varied linearly from 0% to 15% A (v/v) over 40 min with a flow rate of 0.3 mL/min. All samples were run in triplicate.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Relevant properties of the anion-exchange resins used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resin</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>DEAE Cellulose</td>
<td>Whatman</td>
</tr>
<tr>
<td>DEAE Sephacel</td>
<td>GE Healthcare</td>
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<tr>
<td>DEAE Sephadex A-25</td>
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<td>GE Healthcare</td>
</tr>
<tr>
<td>Toyopearl DEAE-650 M</td>
<td>Tosoh Bioscience</td>
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</tbody>
</table>

Figure 1 | Diagram of purification protocol.
5 minutes, followed by 15% to 100% A (v/v) over 60 minutes, and the mobile phase was then held at 100% A for 10 minutes at a flow rate of 0.4 mL/min. The HPLC effluent was delivered into the Z-Spray ES source of a Micromass Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer (Micromass, UK), which was operated in the positive ionization mode at a temperature of 100 °C. Nitrogen was used as the nebulizing gas at a flow rate of 12 L/min and as a desolvation gas at a temperature of 150 °C. The capillary and cone voltages were set at 3.00 kV and 30V, respectively. The software used for the data acquisition and processing was MassLynx NT, version 4.0 (Micromass, UK). The LC system was directly connected to an MS instrument without stream splitting.

**Determination of the yields and purities of the toxins**

**Analysis of the MCs with HPLC**

The analysis of the MCs was performed using an HPLC system with UV detection at a wavelength of 238 nm. The mobile phase consisted of acetonitrile:0.05 M phosphate buffer (pH 3.0) (30:70 v/v) and was run at a flow rate of 1 mL/min. The HPLC system consisted of a 600 pump controller, a 717 plus autosampler, a 2,487 dual λ absorbance detector (Waters, USA) and a TSK-GEL ODS-80Ts column (150 × 4.6 mm) (Tosoh, Japan).

**Toxin yields**

The toxins in each fraction were collected individually and evaporated to dryness under nitrogen gas at a reduced pressure at 40 °C. If the purified toxin was identified as MC-RR or MC-LR, the dried toxin yield was determined using the peak area from the HPLC analysis and comparing this value to that of an authentic standard curve. However, if the corresponding MC standard was not available, the peak areas were compared to a standard curve constructed using MC-LR.

**Toxin purities**

The purity of each MC was determined using the HPLC system at a detection wavelength of 238 nm and was calculated as a peak area percentage of all components in the fractions containing purified toxins.

**RESULTS**

**Extraction and purification of MCs**

The lyophilized cyanobacterial bloom material (50 g) provided an average successive extractive value of approximately 56.28 g (72.56% yield by weight from the lyophilized cyanobacterial material) after acidic-methanol extraction and precipitation with ammonium sulfate. An individual sample of the crude extract was run on each type of DEAE anion-exchange column and eluted with a linear gradient of NaCl. The elution profiles of the extracts loaded onto each column are shown in Figure 2. The chromatographic profile has four peaks at 238 nm (four fractions), designated M-1 to M-4. Similar chromatographic patterns consisting of these four peaks were observed with all of the DEAE chromatography resins that were evaluated. Each of the four peaks was further processed using Sep-Pak C18 cartridges and identified by LC-MS.

**Identification of MCs via LC-ESI-MS analysis**

The compounds in each fraction were identified using LC-MS. The doubly charged \( m/z \) 520 ions and the singly charged \( m/z \) 1,038 ions (Figure 3(a)) indicated the presence of MC-RR in all of the M-1 fractions. The 1,029 \( m/z \) ion confirmed that MC-FR was in the M-2 fraction (Figure 3(b)). Fraction M-3 contained \([\text{Dha}^7]\text{MC-LR}\) with an MS spectrum containing an \( m/z \) 981 in the ESI\(^{+}\) mode (Figure 3(c)). The \( m/z \) 981 ion was further characterized in MS-MS experiments by Somdee et al. (2014). The \([\text{M} + \text{H}]^{+}\) ion at \( m/z \) 1,068 revealed that the M-4 fraction contained MC-WR (Figure 3(d)). In addition, the \( m/z \) 1,029 and 1,068 ions were also confirmed with an MS-MS experiment (spectrum not shown).

**Yields and purities of the MCs**

Four MC variants, MC-RR, MC-FR, \([\text{Dha}^7]\text{MC-LR}\) and MC-WR, were purified from the samples collected from the Bueng Nong Khot reservoir. \([\text{Dha}^7]\text{MC-LR}\) was the major MC variant in the M-3 fraction of the freeze-dried material (Table 2).

The M-1 fraction contained MC-RR, which was obtained with an average yield of 21.14 mg (85–90% purity), whereas
M-2 contained MC-FR, obtained with an average yield of 7.94 mg (79–82% purity); M-3 contained [Dha7]MC-LR, which was obtained with an average yield of 43.38 mg (>90% purity), and M-4 contained MC-WR with an average yield of 6.86 mg (>80% purity).

**DISCUSSION**

A wide variety of methods have been developed for the purification of MCs (Ramanan et al. 2000; Lawton & Edwards 2001; Pyo & Lee 2002; Saito et al. 2002; Aranda-Rodriguez et al. 2003; Barco et al. 2005). The number of steps and procedures used vary widely. In the present study, five DEAE anion-exchange resins with different matrices, exclusion limits and ionic capacities were chosen to evaluate the efficiency of these resins in MC purification. DEAE Sephadex A-25, which contains an ionizable tertiary amine group (DEAE) covalently attached to a cross-linked dextran matrix, produced the best results in MC purification regarding both yield and purity (Table 2), followed by DEAE Cellulose, DEAE Sephacyl, DEAE Sepharose Fast Flow and Toyopearl DEAE. The best performance of the Sephadex resin may be due to the high degree of dextran cross-linking in the matrix and the smaller pore size and higher ionic capacity of the matrix compared with the other types of resins. These properties provide a suitable resin for the purification of small-molecular-weight protein species such as the MCs (the
molecular weight of an MC is approximately 1,000 Dalton) (Fritz & Gjerde 2009). Janson (2011) suggested that a smaller bead matrix would increase the surface area to volume ratio and the number of ionic groups available to bind the proteins. Moreover, a higher ionic capacity results in a larger percentage of charged groups that are available for binding, consistent with the findings of Honey et al. (1994), who suggest that Sephadex is an appropriate resin for

| Table 2 | Yield (mg) and % purity of each MC with different types of DEAE anion exchange columns |
|-----------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Microcystins resin | M-1 (MC-RR) Yield (mg) % Purity | M-2 (MC-FR) Yield (mg) % Purity | M-3 ([Dha]MC-LR) Yield (mg) % Purity | M-4 (MC-WR) Yield (mg) % Purity |
| DEAE Cellulose   | 22.75 86                       | 7.74 79                       | 50.55 92                       | 7.55 81                       |
| DEAE Sephacel    | 18.50 88                       | 7.16 81                       | 46.74 90                       | 6.82 80                       |
| DEAE Sephadex A-25 | 30.16 90              | 10.03 81                      | 53.08 95                       | 7.73 82                       |
| DEAE Sepharose Fast Flow | 17.72 85                 | 8.66 80                       | 34.46 91                       | 6.14 83                       |
| Toyopearl DEAE-650M | 16.56 86                   | 6.12 82                       | 32.05 92                       | 6.06 82                       |
separating low-molecular-weight molecules. For MC purification, Murthy & Capindale (1970) previously used a combination of solvent extraction, dialysis and DEAE-Sephadex A-25 column chromatography to obtain a single MC variant from M. aeruginosa NRC-1 (the MC yield was not reported). In this study, protein precipitation and DEAE Sephadex A-25 chromatography yielded at least four MC variants with significant amounts of the toxins and high purities. This result implies that protein precipitation and DEAE Sephadex A-25 are one of the most effective methods to purify a wide range of MC variants from cyanobacterial material containing a number of MC variants. A complete comparison of these two methods may be further investigated in a future study to determine the most effective method for MC purification.

DEAE cellulose is one of the most common resins used for protein purification (Scopes 1994). DEAE cellulose, containing the same ionizable groups (DEAE) covalently attached to a cellulose matrix, has also been successfully used to purify the toxins. In this study, the lower yield and purity of the DEAE cellulose resin may be due to a lower ionic capacity compared with Sephadex (Table 1), leading to the poor binding ability of the toxins (Janson 2011). However, DEAE-cellulose is now also available in a bead form, DEAE Sephaloc. The Sephaloc resin, DEAE Sepharose Fast Flow, and Toyopearl DEAE resulted in reduced MC yield and purity (Table 2) due to the lower ionic capacity and the larger pore sizes of the cellulose and dextran beads.

Only a few large-scale purifications of MCs have been reported (Saito et al. 2002; Somdee 2010). In this study, a significant amount of cyanobacterial cell material was used, and a substantial quantity of purified toxins was obtained. The results indicated that different types of DEAE anion-exchange chromatography resins were successful in purifying MCs, in agreement with Saito et al. (2002), who obtained both hydrophobic (MC-LW) and hydrophilic MCs (MC-LR) with a one-step column method using a Toyopearl DEAE column.

CONCLUSIONS

Acidic-methanol extraction and precipitation with ammonium sulfate resulted in a crude extract that was suitable for the further purification of MCs. All five of the DEAE anion-exchange resins that were tested performed well in the purification of MCs. The best anion-exchange resin was DEAE Sephadex A-25, which produced the highest yields and purities. However, other DEAE anion-exchange resins are also very useful for the purification of a wide range of MCs from cyanobacterial bloom samples.

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